ANALYZING THE EFFECTS OF PARTIAL GRAVITY ON SKELETAL INTEGRITY

An Honors Fellows Thesis

by

SARAH V. LUNA

Submitted to the Honors Programs Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

April 2010

Majors: Kinesiology and Nutrition

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ABSTRACT

Analyzing the Effects of Partial Gravity on Skeletal Integrity. (April 2010)

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The effect of partial gravity (G) (as on the Lunar surface) on weightbearing bone remains undefined; a new model (the partial G mouse) provides for graded reductions in weightbearing. It was hypothesized that mice exposed to 1/6th G and 1/3rd G (to mimic Lunar weightbearing with full spacesuit) will experience significant reductions in cortical bone mass as compared to ambulatory control animals but that the magnitude of these changes would be less than in 0G mice. Methods: Fifty-eight BALB/cBy female mice were randomly assigned to cage control (1G), "zero-gravity" hindlimb unloaded (0G), 1/6th gravity (G/6), or 1/3rd gravity (G/3) groups for a 21-day suspension protocol. *Ex vivo* pQCT scans (XCT-M Stratec; Norland Corp.) were performed at the midshaft of the excised tibia and humerus to measure volumetric bone mineral density (vBMD), bone mineral content, and cross-sectional geometry. Results: Total body mass significantly decreased (-7.6%) in 0G mice but not in the G/3 or G/6 groups. Relative to the 1G group, cortical shell BMC at the midshaft of the tibia was significantly lower in the 0G, G/6, and G/3 mice but did not differ among the unloaded mice. Cortical area and thickness at the humerus midshaft were significantly lower in the 0G, G/6 and G/3 mice

compared to the 1G mice. Cortical density at the midshaft of the humerus exhibited significant reductions in the 0G and G/6 mice, but not in G/3 mice. There was no significant difference in cortical bone geometry between the 0G, G/6, and G/3 groups at the midshaft of the humerus or tibia. Conclusion: These data suggest that partial weight-bearing (as high as G/3) does not provide enough mechanical loading to prevent the significant deterioration of most cortical bone parameters observed in the 0G non-weightbearing condition.

ACKNOWLEDGMENTS

I would like to recognize the financial support of the National Space and Biomedical Research Institute. This study was funded by the NSBRI via NASA Cooperative Agreement NCC 9-58. This thesis was partially funded by the Honors Programs Office.

Many people contributed to the completion of this work as well. I would like to personally thank my advisor, Dr. Susan A. Bloomfield, for her wonderful guidance. Her valuable insight provided much-needed direction to my work as well as a refined elegance to the ideas presented. I am deeply grateful for her encouragement in all aspects of this project. I would like to acknowledge the invaluable help of Josh Swift, Dr. Liz Greene and Dr. Florence Lima and the entire Bone Biology Lab for so graciously assisting me with this project.

Of course, any mistakes or discrepancies in this work are completely my own and in no way reflect upon the people mentioned above.

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CHAPTER I

INTRODUCTION

Rationale for partial gravity research

> ...we will undertake extended human missions to the moon as early as 2015, with the goal of living and working there for increasingly extended periods of time. — former President George W. Bush [1]

This statement by former President George W. Bush to the National Aeronautics and Space Administration in 2004 inspired a flurry of research in order to determine whether prolonged human inhabitation of the moon would be possible. The deleterious effects of microgravity (0G) on skeletal integrity in humans are well established. Loss of estimated femoral neck strength comparable to that experienced over the lifetime of a Caucasian woman occurs in strong, healthy male astronauts over just six months' time in the harsh space environment; this increases the chance of fractures upon returning to earth [2]. However, it was posited that the one-sixth gravity (G/6) found on the surface of the moon would be sufficient to attenuate the bone wasting seen in microgravity environments. The National Space Biomedical Research Institute (NSBRI) funds specific projects to determine these effects along with specific countermeasures. This specific experiment coordinates with a four-year project in Dr. Susan Bloomfield's laboratory funded by the NSBRI via NASA Cooperative Agreement NCC 9-58.

This thesis follows the style of the journal Bone.

Though rigid and seemingly lifeless, bone tissue constantly restructures based on the forces placed on the body. The gravitational force on Earth places a constant load on bone, which must support the weight of the body on Earth in ambulatory individuals. Major load-bearing bones such as the vertebra, femur and tibia are extremely susceptible to disuse-induced changes when a human experiences the unloading of zero or reduced gravity.

Two types of bone make up these load-bearing bones: cancellous bone and cortical bone. Cancellous bone, also known as trabecular bone, is characterized by a network of interconnected rods and plates. This type of bone has a low density but high amount of surface area, which allows for blood vessels, bone marrow, and connective tissue to be in contact with the bone [3]. Cancellous bone has a higher turnover rate and is found in metaphyseal regions of long bones. In the femoral neck, cancellous bone is a clinically importantbecause it is the site where “hip” fractures occur.

Cortical bone provides the majority of strength and structure to the skeleton. This type of bone is very dense per unit area and is found in the shafts of long bones such as the tibia and humerus and forms a shell around areas rich in cancellous bone, like the femoral neck and vertebral bodies. Cortical bone is nonetheless metabolically active [3]. The inner lining, called the endocortical surface, takes part in bone resorption and formation, while the periosteum on the bone’s outer surface is responsible for

appositional bone growth (gain in total area). See Figure 1 for a graphic representation of cortical bone.

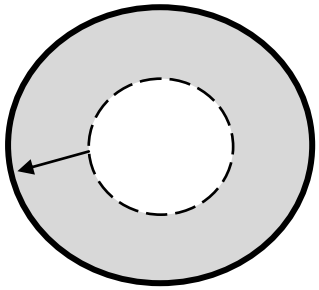

Figure 1. Cross section of cortical bone. Gray shaded area represents cortical area while arrow represents cortical thickness. The bold line represents the periosteal surface while the dashed line represents the endocortical surface.

Microgravity has negative effects on bone

Both animal and human models have shown the negative effects of microgravity (0G) on bone. Hindlimb unloading (tail suspension) in rodents is well established as a model to simulate spaceflight [4-5]. Examples of changes documented in animals exposed to short-duration spaceflight (up to 16 days) include decrements in bone formation [6-9] and reduced mechanical strength [10-13]. In rats, cancellous bone is more affected by hindlimb unloading than cortical bone [14-15] with rare exceptions [16].

Previous studies analyzing the effects of long term spaceflight have studied bone outcomes in humans before and after exposure to 0G (gravity on the International Space Station) and in rodent models subjected to simulated spaceflight (hindlimb unloading)

with control animals allowed regular weightbearing cage activity. Virtually no published studies have documented the effects of simulated Lunar gravity (G/6) on bone strength and architecture, which will be the focus of this thesis project.

Femoral strength in astronauts returning from long-duration spaceflight has been analyzed to some extent. Loss of bone mass and bone density at a rate of about 1.6% per month occurs with long-duration (>4 months) flights, resulting in an increased fracture risk upon return to a 1G environment. Although bone mass is mostly regained over the first year back on earth, analysis by clinical computed tomography (CT) scans revealed that the bones had merely increased in size and not density. This may or may not translate to a recovery of bone strength [2].

Of course, no astronaut willingly donates his femur for mechanical testing; therefore, bone strength cannot be accurately determined in humans. The ethical impossibility of performing mechanical tests on bone from human subjects necessitates the use of complex engineering analyses of CT scan data or animal models. Simulation of microgravity via hindlimb unloading in rodent models illustrates many similarities to the patterns of bone loss observed in humans exposed to spaceflight.

Humans exposed to long-duration spaceflight (> 4 months) exhibit a loss of bone density estimated at 1–2% per month, concentrated mostly in the cancellous compartments of the lower appendicular skeleton [14, 17]. [18]. Finite element models show that

decrements in proximal femoral strength in male crew members over the course of 4-6 month flights on the ISS approach the estimated median *lifetime* loss in strength for Caucasian women [2]. Mathematical modeling of DXA-derived BMD data of 45 ISS crew serving over a total of 56 long-duration ISS missions (4-6 months) show that three years are required to recover from bone loss. [19] Other studies have shown that recovery time is longer than mission time. Bone recovery has been shown to be much slower in mature male rats compared to muscle recovery time [18]; these data raise concerns that skeletal injury may be increased during rehabilitative exercise in humans after prolonged spaceflight or bed rest, when strong muscle contractions might be acting on still weakened bone structures Although there is significant variability in bone loss among individual astronauts, one study by Vico et al. determined that cortical bone loss at the tibia occurred after two months of microgravity [14].

Even among cortical bone, there is variability of the sites of bone loss. DXA data from a study by Lang, et.al. showed significant declines in the volumes of the cortical regions of interest at the femoral neck, trochanter, and total femur (1.2-1.3%/month, $p < 0.05$). The decline in cortical measures was unaccompanied by a change in the outer bone perimeter which suggests that the bone loss occurred at the endosteal surface with no change at the periosteal surface [15]. Bone geometry data can be used to discriminate between fracture and non-fracture populations [20].

Problem summary

Plans to initiate longer term space flight missions, including some to the Lunar surface, have spurred research to answer questions relating to the effects of partial gravity on bone integrity. The deleterious effects of microgravity are known and documented; however, research is needed to show whether partial gravity will attenuate or even protect against the bone losses typically observed during spaceflight.

CHAPTER II

METHODS

Animals and experimental design

Fifty-eight female BALB/cBy mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 3 months of age and allowed to acclimate to their surroundings for 21 days prior to initiation of the study. All animals were housed in a temperature-controlled (23 ± 2ºC) room with a 12-hour light-dark cycle in an American Association for Accreditation of Laboratory Animal Care-accredited animal care facility and were provided standard rodent chow (Harlan Teklad 8604) and water ad-libitum. Animal care and all experimental procedures described in this investigation were conducted in accordance with the Texas A&M University Laboratory Animal Care Committee rules.

Four experimental groups were studied during the 21 days of the study: (1) normal ambulatory cage control (1G, n=11), (2) hindlimb unloaded (0G, n=11), (3) $1/6^{th}$ weightbearing (G/6, n=13), and (4) $1/3^{rd}$ weight bearing (G/3, n=11). Mice were randomly assigned to their respective groups by body mass as recorded one day prior to study initiation in order to achieve groups that at start of experiment that were equivalent in mean body mass. The 0G group was unloaded for 21 days by tail suspension, whereas the 1G animals were allowed normal ambulatory cage activity. Mice in the G/6 and G/3 groups were fitted with harness systems to reduce their weightbearing to $1/6^{th}$

and 1/3rd of their body mass, respectively. All animals were singly housed and monitored for health twice daily.

Calcein injections (15 mg/kg body mass) were given subcutaneously seven and two days prior to sacrifice to label mineralizing bone for histomorphometry analyses. Calcein is a relatively inert tetracycline derivative which binds to circulating calcium in the blood. Bone surface that is actively mineralizing in the 48 hours following each injection incorporates this calcein-labeled calcium. The label fluoresces along that bone surface when histological sections are later viewed under epifluorescent light. This allows for the quantification of the relative bone surface that is actively mineralizing and, by measuring the distance between double labels, the calculation of the bone formed over that seven-day interval before sacrifice [21].

Hindlimb unloaded (0G), G/6, and G/3 animals were anesthetized before removal from tail suspension at the end of the study to prevent any weightbearing by the hindlimbs before tissues were collected. Animals were euthanized by decapitation: left tibiae and humerii were excised, cleaned of soft tissue and wet weights were recorded. Additionally, the distal portions of the right tibiae were stored in 70% ethanol at 4°C for cortical histomorphometry at the tibio-fibular junction (TFJ) region.

Hindlimb unloading

Hindlimb unloading was achieved by tail suspension as previously described [22] and as pictured in Figure 2. Briefly, while the mouse was anesthetized with isoflurane gas (~2.5%) mixed with oxygen, a thin layer of adhesive (Amazing Goop, Eclectic Products, LA) was applied to the proximal half of the tail along the medial and lateral sides. A flat shoelace (~30 cm long) was pressed firmly to the glue and allowed to dry (~5-10 min). Once the mouse is fully conscious, a paper clip was used to attach the animal's tail harness to a swivel apparatus on the wire spanning the top of an 13" x 13" x 13" cage. The height of the animal's hindquarters was adjusted to prevent any contact of the hindlimbs with the cage floor, resulting in approximately a 30° head-down tilt. The forelimbs of the animal maintained contact with the cage bottom, which is a small grid, allowing the mouse to gain traction to move around the cage, allowing access to provided food and water.

Figure 2. Traditional tail suspension model simulates 0G. The tail is supported via a small tail wrap connected to a suspension harness. This model presumes 50% body weight on the forelimbs as is expected with 1G mice. The height of the animal's hindlimbs was adjusted to prevent any contact with the cage bottom. The forelimbs maintained contact with the cage bottom at all times allowing animals access to the entire cage. Figure courtesy of K. Wilkerson.

Partial-weightbearing suspension

Partial-weightbearing was achieved using a well-validated method of simulating the moon's partial gravity (G/6) and Martian gravity (G/3) as developed by Drs. Erika Wagner and Mary Bouxsein [23]. Loading is reduced to a target weight by supporting the mouse's tail and shoulders via a triangular harness attached to the suspending spring. A small piece of wound closure strip adhesive (Henry Schein, Melville, NY) is loosely wrapped at the base of the tail to prevent irritation, and a small piece of standard porous tape (The Kendall Co., Mandsfield, MA) is wrapped around this layer and secured to the harness. The forelimbs are supported by a flexible, moleskin jacket. The two-piece jacket is fabricated from soft moleskin, is secured by Velcro under isoflurane anesthesia. As seen in Figure 3 (below), the harness and tail wrap are connected by an adjustable bead chain and spaced by a hollow metal rod to distribute loading between fore- and hindlimbs.

Titrated suspension is accomplished with a low-modulus aluminum spring. The spring elastic modulus was 0.7 N/m with an initial load of 0.18 N. Daily weighing of suspended animals was accomplished using a modified cage and electronic scale (Ohaus Corp., Pine Brook, NJ). Full body masses were first obtained by briefly hanging the animal on the modified cage positioned over the scale, so as to avoid full weightbearing. Titrated weightbearing was then measured and recorded daily with the animal standing in suspension on the same scale (Figure 3). Adjustments to spring tension were then made

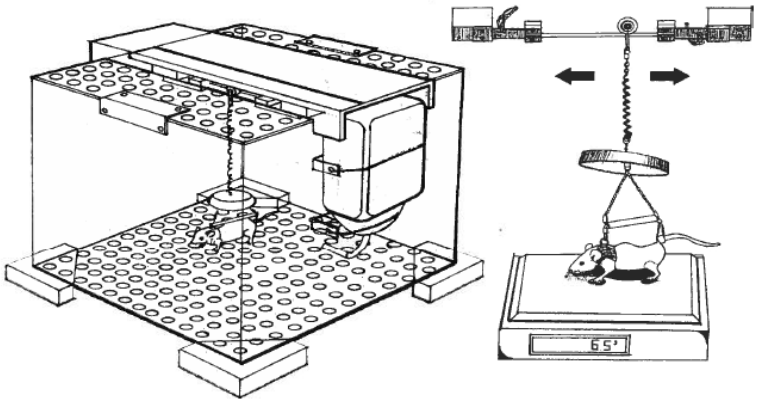

Figure 3. The partial gravity mouse model is a modification of traditional tail suspension. Forelimbs are supported via a moleskin jacket, and the hindlimbs via a small tail wrap; both are connected to a suspension harness. Titration of weight-bearing within ± 0.1 g is achieved via a variable tension spring. Coefficient of variation for partial gravity titration for G/6 was ± 0.16% and G/3 was ± 0.34%. Body weight was checked daily and titrated to within 1% of titrated weight. Model originated by E. Wagner and M. Bouxsein of MIT/Harvard.

as necessary to accommodate changes in body mass and spring stiffness. Animals in the G/6 and G/3 group had their weightbearing titrated back to ~1/6th and 1/3rd, respectively, of their daily body mass.

Correct titration of this model was imperative to the success of this experiment. In order to validate this model of titrated weight bearing, the full weights and partial gravity weights were recorded daily. Actual and target titrated weights were then compared. The objective was to have titrated weights accurate to within 1% of the target weight—an objective which was accomplished with G/6 group at weights within ± 0.16% and G/3 groups within ± 0.34% of the ideal 1/6th or 1/3rd body weight, respectively (Table 1).

Table 1: Accuracy of weight titration of partial gravity mouse model compared to target weight. Values are expressed as mean ± standard deviation.

	G/6	G/3
Day 1	0.88% ± 1.94%	0.84% ± 0.65%
Day 7	0.24% ± 0.21%	0.89% ± 1.44%
Day 14	0.47% ± 0.83%	0.63% ± 0.51%
Day 20	0.18% ± 0.17%	0.37% ± 0.48%
Mean Accuracy over Days 1-20	0.16% ± 0.11%	0.34% ± 0.40%

Peripheral quantitative computed tomography scans

Ex vivo peripheral quantitative computed tomography (pQCT) scans were performed at the proximal and mid-diaphysis of the left tibia and humerus with a Stratec XCT Research-M device (Norland Corp., Fort Atkinson, WI), using a voxel size of 70 μm and a scanning beam thickness of 500 μm. Daily calibration of this machine was performed with a hydroxyapatitie standard cone phantom. Tibiae and humerii were thawed and placed in a 1 mol/L vial filled with phosphate buffered saline (PBS) to maintain hydration during the course of the scan, after which time they were returned to the -80°C freezer. Transverse images of the left tibia were taken at 1.5, 1.75, and 2.0 mm from the proximal tibia plateau, as well as one slice at the midshaft (50% of the total tibia length). Humerii were scanned at the proximal metaphysis (2, 2.25, and 2.5 mm from the proximal plateau) and once at the mid-diaphysis. A standardized analysis for either metaphyseal bone (contour mode 3, peel mode 4, outer threshold of 0.214 g/cm^3, inner

threshold of 0.605 g/cm^3) or diaphyseal bone (contour mode 1, peel mode 2, threshold of 0.605 g/cm^3) was applied to each section.

Values of total and cancellous volumetric bone mineral density (vBMD), total bone mineral content (BMC), total bone area, and marrow area were averaged across the three metaphyseal slices for each outcome variable. For each mid-diaphyseal slice, the outcome variables were cortical vBMD, BMC, cortical bone area, and the polar cross-sectional moment of inertia (CSMI). Machine precision (based on manufacturer's data) is ±3 mg/cm^3 for cancellous vBMD and ±9 mg/cm^3 for cortical vBMD.

Histomorphometry analysis

Undemineralized distal left tibia were subjected to serial dehydration and embedded in methylmethacrylate (Sigma-Aldrich M5, 590-9). Serial cross-sections (120-150 µm) of midshaft cortical bone were cut starting 2.5 mm proximal to the tibio-fibular junction with an Isomet diamond wafer low-speed saw (Buehler, Lake Bluff, IL). Sections were hand ground to reduce thickness (<80 µm) before mounting on glass slides. The histomorphometry analyses were performed using the OsteoMeasure Analysis System, Version 1.3 (OsteoMetrics, Atlanta, GA). Measures of labeled surfaces and interlabel distance were obtained at 200x magnification. Periosteal and endocortical mineral apposition rates (MAR, µm/d), relative mineralizing surface (%MS/BS), and bone formation rates (BFR) were calculated with the formulas below.

$$\text{MAR} = \frac{average\ interlabel\ distance}{time\ between\ labels}$$

$$\%\text{MS/BS} = \frac{single\ labeled\ surface/_2 + double\ labeled\ surface}{surface\ perimeter} \times 100.$$

$$\text{BFR} = MAR \times \% \frac{MS}{BS}.$$

Statistical analyses

All data were expressed as means ± SEM and evaluated using the statistical package SPSS (v.15; Chicago, Ill). Data were analyzed using a one-factor ANOVA to compare group differences between all experimental groups. If statistical significance was found, a Duncan's post-hoc test was used for pair-wise comparisons. For all data, statistical significance was accepted at $p<0.05$.

CHAPTER III

RESULTS

Partial gravity mitigates disuse-associated reductions in body mass

Baseline body masses were not different between cage control and weight-titrated groups (0G, G/6, and G/3). By the end of week 1, the weight-titrated groups had significantly lower body masses than the cage control group. This difference persisted through weeks 2 and 3. After week 2, the hindlimb unloaded group had a body mass significantly lower than its Day 0 body mass. Table 2 shows the recorded body masses for each group. Values are expressed as mean ± standard error. Those letters not shared represent significant differences ($p<0.05$). An asterisk represents a significant difference compared to Day 0 body mass ($p<0.05$). Figure 4 shows the longitudinal changes of body mass over the 21 days. By the end of the 21 day experiment, only the 0G mice had significantly lower body mass compared to the cage control mice showing that partial gravity attenuates disuse-associated decreases in body mass.

Table 2. Change in total body mass (g) of mouse models over 21 days. Group means within a timepoint not sharing the same letter are significantly different (by one-way ANOVA).

	1G (n=11)	0G (n=10)	G/6 (n=13)	G/3 (n=11)
Day 0	23.08 ± 0.31^{b}	22.57 ± 0.27^{b}	22.47 ± 0.36^{b}	22.63 ± 0.49^{b}
Day 7	24.27 ± 0.45^{a}	21.41 ± 0.41^{b}	22.95 ± 0.50^{c}	22.62 ± 0.52^{bc}
Day 14	$24.53 \pm 0.31^{*a}$	$20.49 \pm 0.54^{*c}$	22.20 ± 0.38^{b}	22.09 ± 0.54^{b}
Day 21	24.16 ± 0.43^{a}	$20.81 \pm 0.46^{*b}$	21.56 ± 0.37^{b}	21.76 ± 0.55^{b}
Body Mass Change	1.09 ± 0.22^{a}	-1.76 ± 0.59^{b}	-0.91 ± 0.2^{b}	-0.87 ± 0.32^{b}
% Change	4.7%	-7.6%	-4.0%	-3.8%

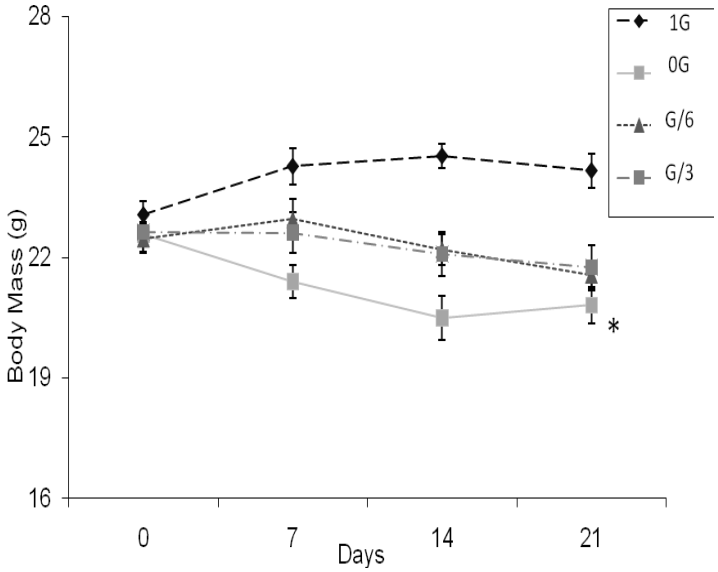

Figure 4. Body mass was significantly reduced from Day 0 in 0G animals only (Asterisk * denotes $p<0.05$ versus Day 0 value). Body mass was maintained over 21 days G/6, G/3 and 1G.

Computed tomography data reveal no protective effect of partial gravity on cancellous bone of tibia or humerus

Partial gravity does not protect against loss of total bone mineral content at the proximal tibia. Table 3 shows the values for total bone mineral content (BMC), total vBMD, total area, and cancellous vBMD.

Table 3: Effects of reduced gravitational loading on structural and geometric properties at the proximal tibia as computed by pQCT scans. Groups not sharing common letters are significantly different ($p<0.05$).

	0G	G/6	G/3	1G
Total BMC (mg)	1.17 ± 0.15^{a}	1.30 ± 0.18^{a}	1.30 ± 0.23^{a}	1.52 ± 0.11^{b}
Total vBMD (mg/mm^3)	486.05 ± 54.64^{a}	502.83 ± 32.50^{ab}	531.09 ± 56.99^{b}	586.30 ± 45.5^{c}
Total Area (mm^2)	0.34 ± 0.04	0.38 ± 0.08	0.35 ± 0.07	0.41 ± 0.06
Cancellous vBMD (mg/mm^3)	224.51 ± 20.38	232.96 ± 20.82	236.45 ± 21.50	269.34 ± 17.43

Both the G/6 and G/3 groups had significantly lower BMCs than the and cage control groups. However, BMC was not significantly different between the hindlimb unloaded group and the G/6 or G/3 groups. Figure 5 illustrates this difference.

The stair-stepping effect expected from having partially titrated weight was finally observed in total vBMD. Bone mineral density of the proximal tibia was significantly different between cage control group and the 0G, G/6, and G/3 groups. The lowest vBMD was observed in the hindlimb unloaded group. The G/6 group mice with weight titrated at $1/6^{th}$ of normal did not differ significantly from either the 0G group or the G/3 group. The G/3 group, however, had significantly higher total vBMD than the hindlimb unloaded group. This can be seen in Figure 5. There were no significant differences between groups for total area or cancellous vBMD.

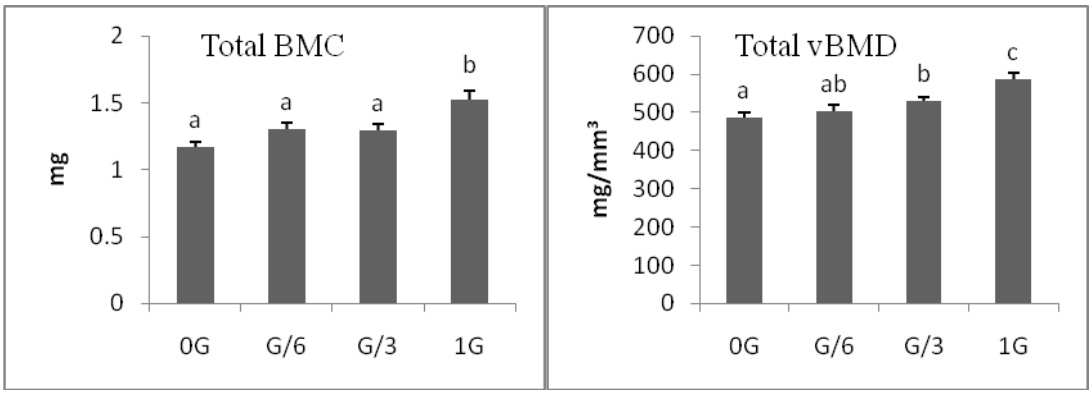

Figure 5. Effects of reduced gravitational loading on proximal tibia. Group means not sharing the same letter are significantly different.

Table 4 shows the values for structural and geometric properties at the proximal humerus as computed by pQCT scans. As with the proximal tibia, outcome variables were total BMC, total vBMD, total area, and cancellous vBMD. There was no significant difference in total BMC between control groups and the hindlimb unloaded group. The G/6 group had significantly lower BMC than the control group. Total vBMD, however, was significantly lower in 0G, G/6, and G/3 groups compared to the control group. Figure 6 shows the results for total vBMD. There were no significant differences between groups for cancellous vBMD, total area, or trabecular area.

Table 4: Effects of reduced gravitational loading on structural and geometric properties at the proximal humerus as computed by pQCT scans. Groups not sharing common letters are significantly different ($p<0.05$).

	1G	0G	G/6	G/3
Total BMC (mg)	0.92 ± 0.07^{a}	0.86 ± 0.04^{ab}	0.81 ± 0.19^{b}	0.85 ± 0.07^{ab}
Total vBMD (mg/mm^3)	1006.93 ± 46.04^{a}	959.05 ± 26.39^{b}	954.48 ± 41.03^{b}	967.90 ± 32.33^{b}
Total Area (mm^2)	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Cancellous vBMD (mg/mm^3)	300.69 ± 22.24	266.69 ± 23.67	274.45 ± 26.85	280.05 ± 17.22
Cancellous BMC (mg)	0.91 ± 0.06	0.90 ± 0.04	0.84 ± 0.18	0.88 ± 0.05
Trabecular Area (mm^2)	0.23 ± 0.02	0.25 ± 0.02	0.23 ± 0.05	0.24 ± 0.02

At the humerus, it is critical to recall that the traditional tail suspension model does not produce a microgravity effect. The humerus (as a forelimb) remains fully weightbearing in this model and therefore should not show significant differences from the 1G model. Consequently, the G/6 mice have the least weightbearing of the models. Figure 6 shows the effects of partial weightbearing on total BMC and total vBMD. As expected, there

are no significant differences between the 0G and 1G mice with the total BMC. The total vBMD data show a significant difference between those groups which suggests a possible systemic effect of unloading. With total BMC, only the G/6 group was significantly different from 1G. There were no differences among unloaded groups for total vBMD and all are significantly lower than 1G.

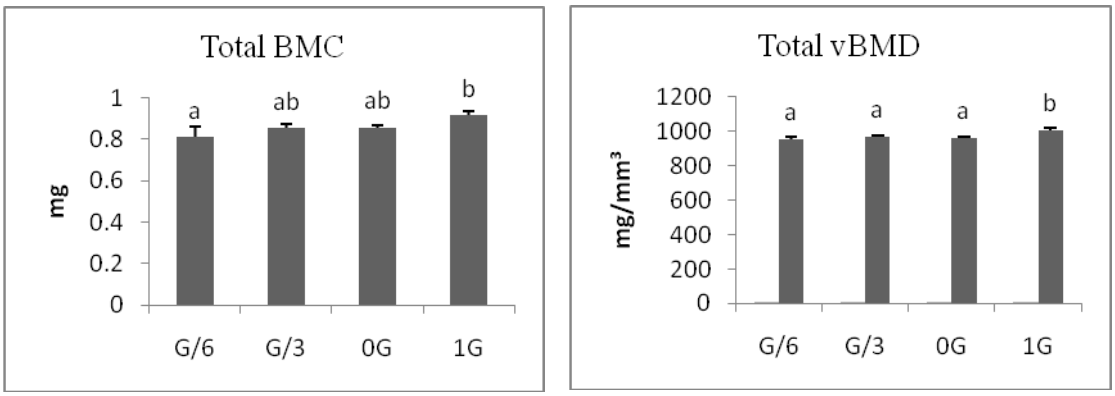

Figure 6. Effects of reduced gravitational loading on proximal humerus. Groups not sharing the same letter are significantly different.

Results are inconsistent for cancellous bone at the tibia and humerus. At the tibia, partial weightbearing lessened the losses of total vBMD seen with complete unloading but had no effect on total BMC. At the humerus, the opposite was observed: partial weighbearing alleviated the negative effect on total BMC but was significantly less than full loading with total vBMD.

Computed tomography data show no difference in cortical bone geometry in tibia

For each mid-diaphyseal slice, the outcome variables were cortical vBMD, BMC, cortical bone area, and the polar cross-sectional moment of inertia (CSMI). Table 5 shows the values for each of the four groups for the midshaft of the tibia. There was no significant difference in the values for cortical BMC, cortical vBMD, cortical area, or cross-sectional moment of inertia between the cage control group and the 0G, G/6, and G/3 groups.

Table 5: Effects of reduced gravitational loading on structural and geometric properties at the midshaft of the tibia as computed by *ex vivo* pQCT scans. No significant differences found between groups.

	1G	0G	G/6	G/3
Total BMC (mg)	0.82 ± 0.06	0.82 ± 0.07	0.77 ± 0.08	0.77 ± 0.08
Total vBMD(mg/mm^3)	1005.57 ± 33.72	1007.56 ± 16.70	1005.11 ± 26.31	1012.90 ± 37.72
Cortical BMC (mg)	0.73 ± 0.06	0.74 ± 0.06	0.69 ± 0.08	0.70 ± 0.08
Cortical vBMD (mg/mm^3)	1327.71 ± 17.15	1320.04 ± 15.49	1316.32 ± 16.70	1325.45 ± 19.51
Cortical Area (mm^2)	0.55 ± 0.04	0.56 ± 0.05	0.52 ± 0.05	0.53 ± 0.05
CSMI (mg/mm^4)	0.16 ± 0.03	0.15 ± 0.02	0.14 ± 0.03	0.15 ± 0.02

Computed tomography does not show protective effect of partial gravity in cortical bone geometry in humerus

Table 6 shows the effects of gravitational loading on the midshaft of the humerus. The 0G, G/6, and G/3 groups had significantly lower cortical contents than the cage control

group. However, there was no difference between the unloaded groups. Figure 7 illustrates cortical content. Cortical density was also affected by loading. The hindlimb unloaded group had significantly lower cortical density than the cage control group, as did the G/6 group; however, the G/3 group did not significantly differ from either the 1G, 0G, or G/6 groups. Figure 7 compares the cortical density values for the midshaft of the humerus.

Table 6: Effects of reduced gravitational loading on structural and geometric properties at the midshaft of the humerus as computed by *ex vivo* pQCT scans. Groups not sharing common letters are significantly different ($p<0.05$).

	1G	0G	G/6	G/3
Total BMC (mg)	0.79 ± 0.02	0.71 ± 0.03	0.66 ± 0.16	0.70 ± 0.06
Total vBMD (mg/mm^3)	941.96 ± 22.99	881.86 ± 19.89	912.65 ± 53.77	903.99 ± 48.85
Total Area (mm^2)	0.84 ± 0.03	0.80 ± 0.03	0.73 ± 0.19	0.78 ± 0.04
Cortical BMC (mg)	0.69 ± 0.02[a]	0.61 ± 0.02[b]	0.57 ± 0.14[b]	0.60 ± 0.06[b]
Cortical vBMD (mg/mm^3)	1325.71 ± 18.23[a]	1293.91 ± 20.42[b]	1287.81 ± 54.97[b]	1302.30 ± 33.16[ab]
Cortical Area (mm^2)	0.52 ± 0.02[a]	0.47 ± 0.01[b]	0.44 ± 0.10[b]	0.47 ± 0.04[b]
Cortical Thickness (mm)	0.20 ± 0.01[a]	0.18 ± 0.00[b]	0.18 ± 0.02[b]	0.18 ± 0.01[b]
CSMI (mg/mm^4)	0.16 ± 0.01[a]	0.15 0.01[ab]	0.13 ± 0.04[b]	0.14 ± 0.01[b]

Cortical area was also affected by loading. The hindlimb unloaded group had significantly lower cortical density than the cage control group. There was no significant difference between the hindlimb unloaded group and the G/6 and G/3 groups. Figure 7 compares the cortical area values for the midshaft of the humerus.

Cortical thickness was significantly lower in the 0G, G/6, and G/3 groups compared to the cage control group. Figure 7 shows cortical thickness for the midshaft of the humerus. Cross-sectional moment of inertia was significantly lower in the G/6 and G/3 groups compared to both the 1G and 0G groups (Table 6).

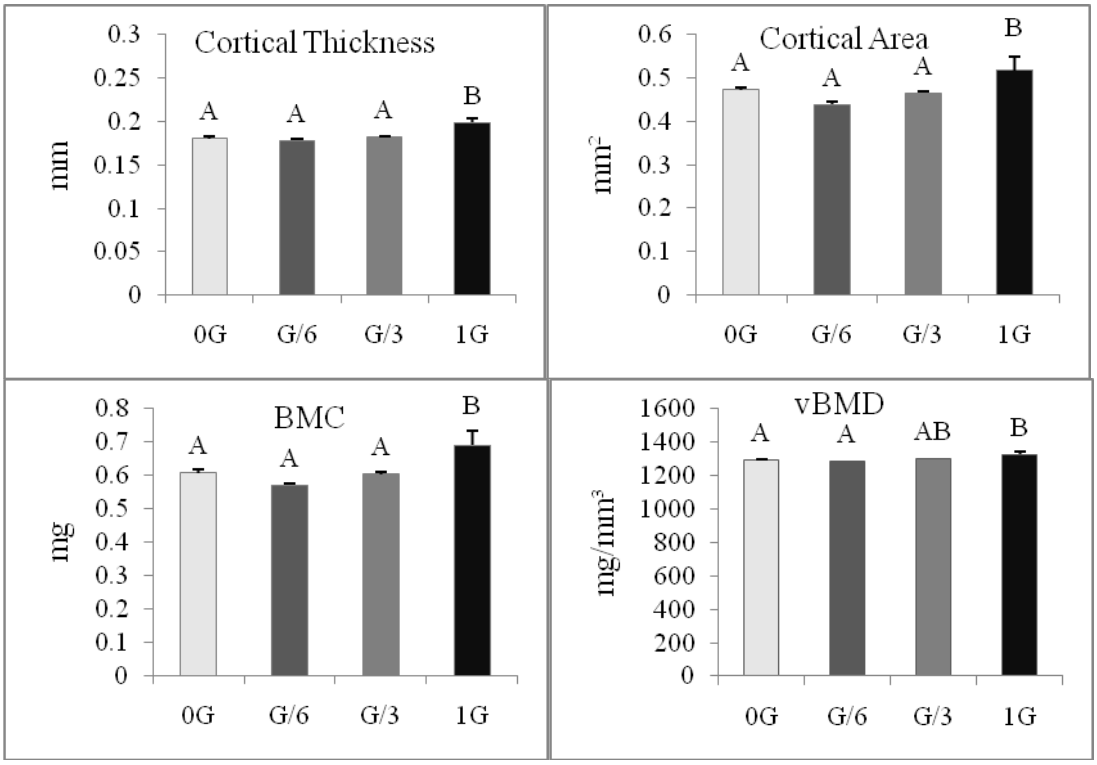

Figure 7. Effects of reduced gravitational loading on midshaft of humerus. Those group means not sharing the same letter are significantly different from each other ($p < 0.05$).

Histomorphometry data show inconsistent results

On days 14 and 19 of the study, mice were given intraperitoneal calcein injections. As mentioned previously, calcein labeling fluoresces and allows for the quantification of

mineralizing surface. Figure 8 shows the fluorescing calcein label in newly formed bone. Mineralizing surface can be thought of as the quantity of osteoblast teams on a bone site. It is expressed as a percent: mineralizing surface over total bone surface (MS/BS). Mineral apposition rate (MAR) analyzes the distance between double label (when it occurs) and is a measure of osteoblast vigor. Bone formation rate is the product of MS/BS and MAR.

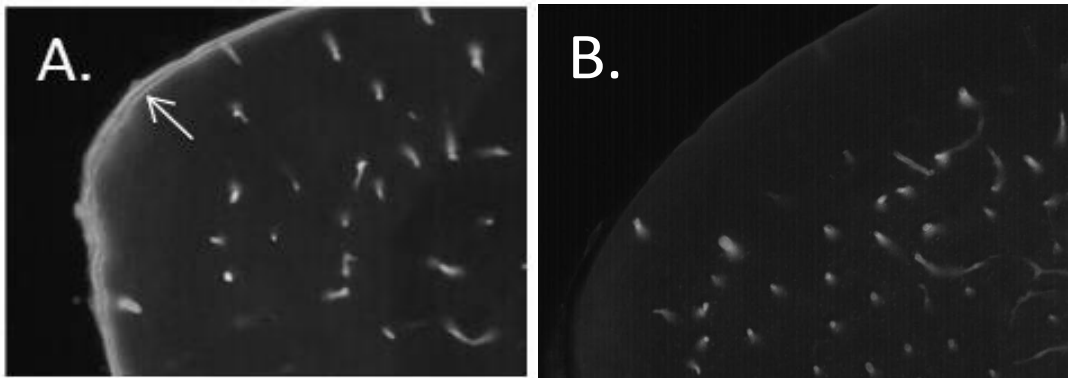

Figure 8. Image of calcein labeling on the periosteal surface of a cortical bone section taken near the mid-diaphysis of the tibia (rodent). (A) clearly marked double labeling (see arrow); (B) absence of labels on periosteal surface.

Mineralizing surface on the endocortical surface of the tibia was significantly lower in the hindlimb unloaded group (Table 7), the G/6 group, and the G/3 group compared to the control groups. There was no significant difference in mineral apposition rate between 1G, 0G, or G/6 groups. The G/3 group had a significantly lower mineral apposition rate than the control group. Bone formation rate differed significantly between the cage control group and 0G and G/3 groups. The Lunar group did not differ significantly with the control group.

Table 7 shows the histomorphometry data for the periosteal and endocortical surfaces of the tibia. Mineralizing surface of the periosteal surface did not differ significantly between the 1G, 0G, G/6, and G/3 groups. There was no significant difference seen in mineralizing apposition rate between the groups. There was no significant difference between the groups for bone formation rate.

Table 7. Histomorphometry data for the endocortical and periosteal surfaces of the midshaft of the tibia. Groups not sharing common letters are significantly different ($p<0.05$). No significant differences between periosteal groups.

	1G	**0G**	**G/6**	**G/3**
		Endocortical		
MS/BS	17.33 ± 4.66^{a}	5.24 ± 2.62^{b}	6.61 ± 2.26^{b}	4.65 ± 2.20^{b}
MAR	0.36 ± 0.05^{a}	0.17 ± 0.07^{ab}	0.26 ± 0.09^{ab}	0.12 ± 0.06^{b}
BFR	28.75 ± 9.88^{a}	6.50 ± 3.71^{b}	13.13 ± 5.77^{ab}	5.59 ± 3.03^{b}
		Periosteal		
MS/BS	3.64 ± 1.55	2.58 ± 1.07	1.74 ± 0.79	0.68 ± 0.20
MAR	0.14 ± 0.06	0.16 ± 0.09	0.06 ± 0.03	0.11 ± 0.06
BFR	4.72 ± 2.99	3.09 ± 1.87	1.15 ± 0.62	0.44 ± 0.23

As shown in Figure 9, there were no significant differences between groups for any of the periosteal parameters. Endocortical data shows conflicting results with some weightbearing being protective of bone formation rate. MS/BS is depressed for all unloaded groups with no differences between those groups. MAR and BFR show inconsistent results.

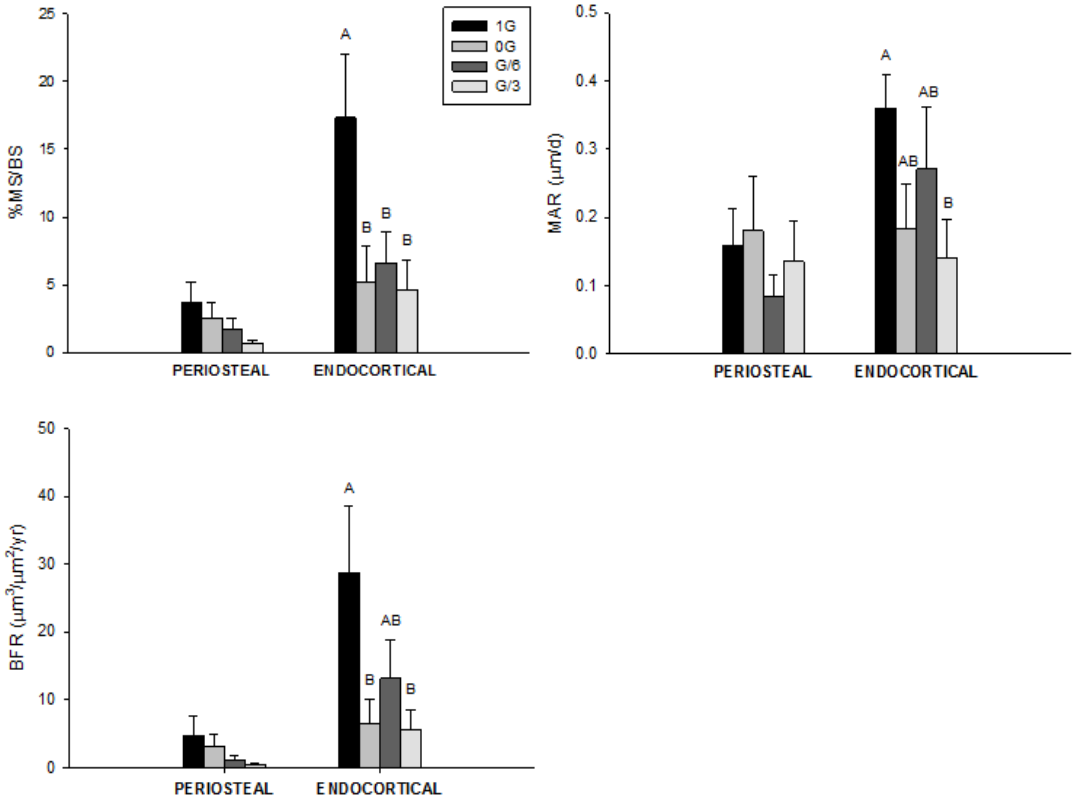

Figure 9. Effects of 21 days of normal ambulation (1G) hindlimb unloading (0G), G/6, or G/3 gravitational weight-bearing on periosteal and endocortical surface dynamic histomorphometry analyses measured at the tibial mid-diaphysis. Those groups not sharing the same letter for respective surface measures are significantly different from each other ($p<0.05$). Group means with no labels are not significantly different.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Discussion

The data collected from this experiment were not wholly consistent with previous data nor did they support the hypothesis that partial gravity would have protective effects on the negative changes seen with microgravity.

It is worth noting that the pQCT data on the cortical bone in the tibia did not show any differences between any of the groups. This is consistent with most other rodent studies. The pQCT method is adequate for showing significant changes on rat bones; however, smaller mouse bones may require more sensitive scanning procedures. One potential improvement for this study is the use of the micro CT method for the mouse bones which has a higher resolution and could potentially differentiate a significant change.

The cortical humerus data also presents an amusing challenge. Recalling the traditional model of tail suspension, one sees that the humerus (being a forelimb) is not unloaded at all. Because of the setup of the tail suspension model the 0G group and the 1G group are both fully loaded at the humerus. The cortical data, however, shows a significantly lower cortical thickness, area, content, and density in the 0G group compared to the fully loaded group. Since the tail suspension model presumes 50% body weight on the fore-limbs (as is expected with 1G mice), these results suggest that this model of simulated

microgravity has some sort of systemic effect on bone. A study by Wronski and Morey in 1983 showed similar effects in the tibia and humeri of unloaded rats [24]. If decrements in bone integrity are thought to be merely caused by mechanical unloading, then only the non-weightbearing bones of the unloaded subjects would exhibit negative effects. However, the weightbearing humeri showed effects that were not significantly different from the unloaded tibia which suggests the interplay of other factors. For example, extended hindlimb unloading increases the circulating levels of the stress hormone cortisol which may have a detrimental effect on bone.

The final oddity to be reconciled is the apparent disconnect between the histomorphometry data and the pQCT data. The pQCT data show no protective effect of partial loading on either humerus or tibia. The histomorphometry data give a muddled picture but tend to show a protective effect of partial gravity on the endocortical surface of the tibia. There are a few explanations. Mentioned previously was the possibility that conducting pQCT would not have the sensitivity to detect significant changes in mice bones. Perhaps a higher resolution method would show a protective change comparable to the histomorphometry data. Another possible explanation is that the study design of 21 days is not long enough to show the effects of a depressed bone formation rate in bone geometry. Additionally, significant depression at endocortical surface may not be sufficient to show depression in bone geometry without accompanying depression at the periosteal site. Changes seen in histomorphometry data showed inconsistent effects with 0G and G/6 at the endocortical site.

Conclusions

After 21 days, body mass was significantly lower in the 0G compared to the 1G. Body mass change was significantly lower in all reduced weight-bearing groups compared to the 1G group. Body mass was maintained over 21 days in G/6, G/3 and 1G groups. pQCT data of the midshaft of the tibia shows no significant changes in bone geometry between unloaded, partially loaded, and fully loaded groups. Humeral pQCT data reveals a significant difference between the 1G and 0G groups in all cortical bone parameters tested suggesting that the tail suspension model reduces load on the forelimbs. One-third gravity is insufficient to protect against loss of cortical thickness, area, and content but does protect against significant losses in volumetric bone density in the midshaft of the humerus. There were no significant differences in periosteal mineralization surface, mineral apposition rate, or bone formation rate between the 0G, G/6, G/3 and 1G groups. Endocortical parameters differed significantly between the 1G group and the unloaded groups. However, partial weightbearing did not protect against lower mineralization surface, mineral apposition rate or bone formation rate with the exception of the G/3 group. Most of these data suggest that partial weightbearing up to G/3 does not prevent the deleterious changes in indices of cortical bone formation activity observed with traditional tail suspension. Astronauts in a partial gravity environment, therefore, would still need countermeasures to address the loss of bone integrity.

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